APPLICATION

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TITLE: **OVERCOMING DAPA AMINOTRANSFERASE BOTTLENECKS IN BIOTIN VITAMERS BIOSYNTHESIS**

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OVERCOMING DAPA AMINOTRANSFERASE BOTTLENECKS IN BIOTIN VITAMERS BIOSYNTHESIS

Background of the Invention

The present invention is in the general field of the biosynthesis of biotin vitamers.

Biotin biosynthesis in *Escherchia coli* and *Bacillus sphaericus* has been studied at both the biochemical and molecular biological levels (DeMoll, 1996. *In* F.C. Neidhardt et al., (eds.) *E. coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, Second edition ed., vol 1., pp. 704-709, ASM Press, Washington, D.C.; Perkins et al., *In* A.L. Sonenshein et al. (eds.), *In Bacillus subtilis* and Other Gram Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics, pp. 319-334, American Society for Microbiology, Washington, D.C.; Eisenberg, 1987. *In* F. Neidhardt et al. (eds.), *E. coli* and *Salmonella typhimurium*, pp. 544-550. American Society for Microbiology, Washington, D.C.; Cronan, Cell 58:427-429, 1989, Izumi et al., Agric. Biol. Chem. 45:1983-1989, 1981; Gloeckler et al., Gene 87:63-70, 1990), although some steps and components in biotin synthesis remain to be elucidated (Ohshiro et al., Biosci. Biotech. Biochem. 58:1738-1741, 1994; Ifuku et al., Eur. J. Biochem. 224:173-178, 1994; Florentin et al., C.R. Acad. Sci. Paris 317:485-488, 1994; Birch et al., J. Biol. Chem. 270:19158-19165, 1995; Sanyal et al., Biochemistry 33:3625-3631, 1995). Several enzymes involved in the conversion of pimeloyl-CoA to biotin have been isolated and characterized from both of these bacterial species (Ploux et al., Biochem. J. 283:327-321, 1992; Izumi et al., Agric. Biol. Chem. 45:1983-1989, 1981; Eisenberg, *supra*, Huang et al., Biochemistry 34:10985-10995, 1995). KAPA synthase, the product of *bioF*, catalyzes the conversion of pimeloyl-CoA

and alanine to 8-amino-7-ketopelargonic acid (KAPA). DAPA aminotransferase, the product of *bioA*, then transfers an amino group from a donor to KAPA yielding 7,8-diaminopelargonic acid (DAPA). Dethiobiotin synthetase (*bioD*) catalyzes the closure of the ureido-ring to produce dethiobiotin (DTB), and finally the product of *bioB*, biotin synthase, functions together with a number of other components including flavodoxin (Birch et al., *supra*; Ifuku et al., *supra*) S-adenosylmethionine (SAM) (Florentin, C.R. Acad. Sci. Paris 317:485-488, 1994; Ohshiro et al., *supra*; Sanyal et al., *supra*; Birch et al., *supra*) ferrodoxin $NADP^+$ reductase (Birch et al., *supra*; Sanyal et al., Arch. Biochem. Biophys. 326:48-56, 1996) and possibly cysteine (Florentin, C.R. Acad. Sci. Paris 317:485-488, 1994; Birch et al., *supra*; Sanyal et al., *supra*) to convert dethiobiotin to biotin. The compounds KAPA, DAPA, DTB, and biotin are collectively or singly referred to as vitamers or biotin vitamers.

In *E. coli* the genes that encode these enzymes are located in two divergently transcribed operons, controlled by a single operator that interacts with the BirA repressor (Cronan, Cell 58:427-429, 1989). In *B. sphaericus*, the genes are located in two separate operons (Gloeckler et al., *supra*. The early steps of the pathway, those involved in the synthesis of pimeloyl-CoA, are less well understood (Ifuku et al., Eur. J. Biochem. 224:173-178, 1994; Sanyal et al., J. Am. Chem. Soc. 116:2637-2638, 1994). *B. sphaericus* contains an enzyme, pimeloyl-CoA synthetase (*bioW*) that converts pimelic acid to pimeloyl CoA (Gloeckler et al., Gene 87:63-70, 1990), (Ploux et al., Biochem. J. 287:685-690, 1992). *E. coli* lacks this enzyme and cannot use pimelic acid as an intermediate in biotin synthesis (Gloeckler et al., *supra*; Ifuku et al., Eur. J. Biochem.

224:173-178, 1994; Sanyal et al., J. Am. Chem. Soc. 116:2637-2638, 1994). *E. coli* contains two genes, *bioC* which is located in the *bio* operon and *bioH* which is unlinked to the other *bio* genes, that both appear to be involved in the early steps of biotin biosynthesis leading up to pimeloyl-CoA, but their exact roles are unknown (Eisenberg, *supra*; Lemoine et al., Mol. Micro. 19:645-647, 1996).

B. subtilis contains homologs of the *E. coli* and *B. sphaericus bioA*, *bioB*, *bioD*, and *bioF* genes. These four genes along with a homolog of the *B. sphaericus bioW* gene are arranged in a single operon in the order *bioWAFDB*, and are followed by two additional genes, *bioI* and *orf2* (Bower et al., J. Bacteriol. 178:4122-4130, 1996). *bioI* and *orf2* are generally dissimilar to other known biotin biosynthetic genes. The *bioI* gene encodes a protein with similarity to cytochrome P450s and is able to complement mutations in either *E. coli bioC or bioH* (Bower et al., *supra*. Mutations in *bioI* cause *B. subtilis* to grow poorly in the absence of biotin. The bradytroph phenotype of *bioI* mutants can be overcome by pimelic acid, suggesting that the product of *bioI* functions at a step prior to pimelic acid synthesis (Bower et al., *supra*.

The *B. subtilis bio* operon is preceded by a putative vegetative promoter sequence and contains, just downstream, a region of dyad symmetry with homology to the *bio* regulatory region of *B. sphaericus* (Bower et al., *supra*. Analysis of a *bioW-lacZ* translational fusion indicates that expression of the biotin operon is regulated by biotin and the *B. subtilis birA* gene. Strains deregulated for biotin synthesis can be engineered by replacing the promoter and regulatory region with a constitutive promoter as described in European Patent Application 0635572 A2, incorporated

herein by reference. Production of biotin and biotin vitamers can be further improved by integration and amplification of the deregulated genes in the *B. subtilis* chromosome. Strain BI282, in European Patent Application 0635572 A2, herein incorporated by reference, is an example of such a strain.

Summary of the Invention

We have found that the conversion of KAPA to DAPA is a serious bottleneck in the biosynthesis of biotin using engineered cells that are fed pimelic acid. As other controls on biotin biosynthesis are removed, the KAPA to DAPA conversion is unable to keep pace with KAPA production, resulting in a build-up of KAPA, without a concomitant increase in the final product. We have also discovered that an important component of the bottleneck is the availability and identity of the amino donor used in the KAPA to DAPA conversion. In general, providing adequate quantities of the amino donor is an important strategy for overcoming the bottleneck. Moreover, a DAPA aminotransferase able to use lysine and related compounds as a source of the amino group to be transfered in the reaction which produces DAPA from KAPA, can significantly improve biosynthetic yields of the downstream biotin vitamers, especially dethiobiotin (DTB).

Although we do not wish to be limited to one specific explanation for our finding to the exclusion of other factors, it appears that providing higher levels of an amino donor which can be used by the available aminotransferase substantially ameliorates the bottleneck discussed above. For example, bacterial production of the biotin vitamers by bacteria whose DAPA aminotransferase uses lysine as an amino donor can be dramatically improved by making sufficient lysine available, either by including it

in the fermentation medium or by deregulating the lysine biosynthetic pathway. Such a strategy can also be applied to the use of DAPA aminotransferases of *B. subtilis* and close relatives, including members of the cluster of *Bacillus* spp. represented by *B. subtilis*. The cluster includes, e.g., *B. subtilis, B. pumilus, B. licheniformis, B. amyloliquefaciens, B. megaterium, B. cereus* and *B. thuringiensis*. The members of the *B. subtilis* cluster are genetically and metabolically divergent from the more distantly related *Bacillus* spp. of clusters represented by *B. sphaericus* and *B. stearothermophilus* (Priest, *In Bacillus subtilis* and Other Gram-Positive Bacteria, *supra* pp. 3-16, hereby incorporated by reference; Stackebrant, et al. *J. Gen. Micro.* 133:2523-2529, 1987, hereby incorporated by reference).

Accordingly, one aspect of the invention generally features a method of biosynthesizing (e.g., enzymatically or in fermentations using engineered cells) a biotin vitamer by culturing a bacterium that includes a lysine-utilizing DAPA aminotransferase in an environment enriched in lysine, lysine precursor(s), or analog(s). The desired biotin vitamer is then recovered from the environment. The ability of an amino donor to be used with a given aminotransferase may be evaluated in any appropriate assay, including but not limited to a bioassay based on that described by Eisenberg and Stoner (1971, *infra*) in which a DAPA sensitive strain of *E. coli* is used to measure DAPA aminotransferase activity. Typically, the bacterium will also be deregulated with respect to one or more biotin synthetic pathway steps, e.g., as described in EP 635572, incorporated above. The DAPA aminotransferase may be produced by the cell's wild-type genetic material, by exogenous nucleic acid introduced into the cell, or both.

As used herein, a "lysine-utilizing DAPA aminotransferase" means a DAPA aminotransferase capable of converting 8-amino-7-ketopelargonic acid (KAPA) to diaminopelargonic acid (DAPA) utilizing lysine or a compound that is converted to lysine or a compound that can substitute for lysine as the amino donor.

As used herein, an "environment enriched for" means a bacterial culture in which the concentration of the indicated molecule is greater than that found under standard culture conditions, and greater than is necessary to avoid limiting cell growth in the absence of biotin vitamer overproduction.

The biotin vitamer product to be recovered and purified can be biotin, dethiobiotin, or diaminopelargonic acid (DAPA). When dethiobiotin or DAPA is recovered, the method may further include the step of converting the recovered dethiobiotin or DAPA to biotin.

In another aspect of the invention a bacterial strain is also engineered to overcome the KAPA-to-DAPA bottleneck by overproducing a DAPA aminotransferase capable of transferring an amino group from an amino donor to 8-amino-7-ketopelargonic acid (KAPA). In a preferred embodiment of this aspect of the invention, the bacterial strain is further engineered to overproduce the biotin vitamer by deregulation of a biotin biosynthetic step other than the KAPA-DAPA step.

To further circumvent the KAPA-to-DAPA bottleneck, the strain may be further engineered to produce multiple DAPA-aminotransferases, relying on different amino donors (e.g., lysine and SAM). These activities may be assayed and distinguished as described in detail below. Briefly, the level of KAPA-to-DAPA conversion may be measured by vitamer bioassays and bioautography of products from bacteria grown

in the presence of lysine, methionine, or lysine and methionine.

As used herein, "SAM-utilizing DAPA amino transferase" means a DAPA aminotransferase capable of converting 8-amino-7-ketopelargonic acid (KAPA) to diaminopelargonic acid (DAPA) utilizing S-adenosylmethionine (SAM) or a compound that is converted to SAM or a compound that can substitute for SAM as the amino donor.

In other embodiments, methionine and lysine, or their analogs are added to the medium.

One way to provide a lysine-rich environment is to enrich the culture with lysine or a lysine homolog that can donate an amino group to KAPA in the DAPA aminotransferase reaction. Lysine homologs include lysine, (S)-2-aminoethyl-L-cysteine (AEC) and other lysine homologs that can serve as amino donors for a DAPA aminotransferase. Another way to provide a lysine-rich environment is to deregulate the bacterium with respect to lysine production by mutating or engineering it to significantly reduce wild-type control over lysine production. For example, deregulation of a lysine synthetic step includes reducing or removing regulation of transcriptional or other expressional control of a lysine synthetic enzyme, or modification of a lysine synthetic enzyme to reduce or remove control over lysine biosynthesis. Deregulation also includes overproducing compounds which are starting materials in the lysine synthetic pathway, and inhibiting biodegradation of lysine (Amino Acids: Biosynthesis and Genetic Regulations, E. Hermann and R. Somerville (eds.) Addison Wesley, Reading, MA 1983, pp. 147-172, 213-244, 417).

Deregulation of a biotin synthetic step includes reducing or removing regulation of transcriptional or other expressional control of a biotin synthetic enzyme, or

modification of a biotin synthetic enzyme to reduce or remove control over the enzyme-catalyzed biotin synthetic reaction. It can also include overproducing compounds which are starting materials in the biotin synthetic pathway, and inhibiting biodegradation of a desired biotin vitamer.

Bacteria can be engineered by intentionally and specifically altering the wild-type genome to produce a desired biosynthetic phenotype -- e.g., to synthesize more lysine than the corresponding wild-type, unengineered organism, or to remove a bottleneck in the biotin biosynthetic pathway.

Conversion of DTB to biotin may be by any means including but not limited to biochemical conversion of DTB to biotin, feeding DTB to bacteria engineered for the bioconversion of DTB to biotin (Fujisawa et al., Biosci. Biotech. Biochem. 57:740-744, 1993), *in vitro* synthesis of biotin from DTB (Birch et al., J. Biol. Chem. 270:19158-19165, 1995; Fujisawa et al., FEMS Microbiology Letters 110:1-4, 1993; Ifuku et al., Biosci. Biotech. Biochem. 56:1780-1785, 1992; Birch, WO 94/08023) or chemical synthesis.

Brief Description of the Drawings

Figure 1 is a representation of data showing the effect of KAPA concentration on *B. subtilis* DAPA aminotransferase activity.

Figure 2 is a reciprocal plot of initial velocity data for *B. subtilis* DAPA aminotransferase in the presence of varying concentrations of KAPA.

Figure 3 is a representation of results from a bioautography of fermentation broths of different bacterial strains with lysine and methionine or with or without lysine, as described in Table 5.

Figure 4 is a diagram of the *B. subtilis* biosynthetic pathway for lysine and related compounds.

Description of Tables

Table 1 is a representation of the data of a DAPA aminotransferase assay of an extract of BI611 by addition of potential amino donors to the reaction mix.

Table 2 is a representation of the results of a DAPA aminotransferase assay of an extract of BI611 after addition of lysine or lysine-related compounds to the reaction mix.

Table 3 is a representation of biotin and vitamer production of BI282 and BI603 grown in bench scale fermenters in the presence of 6 g lysine/liter.

Table 4 is a representation of biotin and vitamer production of BI282, BI96, and BI90 grown in bench scale fermentors in the presence of 3 g methionine/liter in the batch and feed.

Table 5A-5B represents biotin and vitamer production from strains BI603 and BI90 grown in bench scale fermentors in the presence or absence of 6 g lysine/liter and 3 g methionine/liter.

Table 6 is a representation of results of an assay of biotin and vitamer production using different lysine feed regimens.

Table 7 lists known *B. subtilis* lysine-deregulated mutants.

Table 8 is a representation of results of an assay of biotin and vitamer production of bacterial strains resistant to AEC grown in the presence of pimelic acid.

Appendix I describes a composition of medium for biotin and vitamer production in bench scale fermentors.

Appendix II describes an avidin-HABA displacement assay for biotin and DTB.

Description of the Preferred Embodiments

A bottleneck in KAPA-to-DAPA conversion occurs during pimelic acid-fed fermentations of *B. subtilis*. In the experiments described below, we discovered that in *B. subtilis*, DAPA aminotransferase uses lysine as an amino donor, in contrast to S-adenosylmethionine (SAM), the compound that serves as the amino donor for DAPA aminotransferases of *B. sphaericus* (Izumi et al., Agric. Biol. Chem. 45:1983-1989, 1981), *Brevibacterium divaricatum*, *Salmonella typhimirium*, *Aerobacter aerogenes*, *Bacillus roseus*, *Micrococcus roseus*, and *Sarcina marginata* (Izumi et al., Agr. Biol. Chem. 39:175-181, 1975), *E. coli* (Eisenberg et al., J. Bacteriol. 108:1135-1140, 1971), and *S. marcescens*.

In *E. coli* and *B. sphaericus*, the conversion of KAPA to DAPA is catalyzed by DAPA aminotransferase, the product of the *bioA* gene, which utilizes SAM and KAPA as substrates (Eisenberg et al., J. Bacteriol. 108:1135-1140, 1971; Izumi et al., Agric. Biol. Chem. 45:1983-1989, 1981; Stoner et al., J. Biol. Chem. 250:4037-4043, 1975; Stoner et al., J. Biol. Chem. 250:4029-4036). It had been assumed that the reaction was similar in *B. subtilis* since the *B. subtilis* aminotransferase is 33% homologous with the *E. coli* enzyme and can complement *bioA* mutants in *E. coli*. However, *in vitro* assays of the *B. subtilis* enzyme led to our surprising discovery that lysine is an amino donor for the *B. subtilis* DAPA aminotransferase. Furthermore, the addition of lysine (2-10 g/l) to the fermentation medium of *B. subtilis* biotin production strains such as BI282 reduced the amount of KAPA produced and led to the accumulation of significant quantities of dethiobiotin (DTB). Various fermentative or chemical methods can then be used to convert DTB to biotin.

The observation that SAM was not a significant amino donor for the *B. subtilis* DAPA aminotransferase provided the clue to overcome this bottleneck. A search was made for the real amino donor. After testing 26 different amino acids and related compounds, only lysine was found to dramatically stimulate the *in vitro* conversion of KAPA to DAPA by the *B subtilis* DAPA aminotransferase. In subsequent testing, D- and L-lysine and the lysine analog, (S)-2-aminoethyl-L-cysteine (AEC), were found to function as amino donors with the *B. subtilis* enzyme. Thus, any of these, in any combination, may be used in the invention. Although there are other known aminotransferases that use lysine as an amino donor (Tobin *et al.*, 1991. *J. Bacteriol.* 173:6223-6229; Coque *et al.*, 1991. *J. Bacteriol.* 173:6258-6264; Soda et al., 1968. Biochemistry 7:4102-4109; Soda and Misono, 1968, Biochemistry 7:4110-4119; Schmidt et al., 1988, FEMS Microbiol. Lett. 49:203; Lowe and Rowe, 1986. Mol. Biochem. Parasitol. 21:65), no other known DAPA aminotransferase uses lysine. Both the *E. coli* and the *B. sphaericus* BioA enzymes use SAM (Eisenberg et al., J. Bacteriol. 108:1135-1140, 1971; Izumi et al., Agric. Biol. Chem. 45:1983-1989, 1981; Stoner et al., J. Biol. Chem. 250:4037-4043, 1975; Stoner et al., J. Biol. Chem. 250:4029-4036).

Characterization of the *B. subtilis* DAPA aminotransferase indicated that the K_m for lysine was high and, it was substrate inhibited by KAPA. We conclude that the KAPA to DAPA bottleneck was caused by insufficient lysine or an unfavorable ratio of KAPA/lysine, and that the addition of lysine to the fermentation medium could overcome the block.

When fermented with added lysine (6 g/l), as well as pimelic acid (1 g/l), the engineered *B.subtilis* strain BI282 (bio::$[\mathbf{P}_{15}bio]_{7-8}$) showed a dramatic increase (>10-fold) in

DTB production. Under these fermentation conditions, BI282 produced about 300-700 mg/l of DTB. Depending on the exact fermentation medium and conditions, nearly all of the KAPA could be converted to DTB. Also, fermentation of strain BI90, a derivative of BI282 that contains a single-copy cassette with the *E.coli bioA* gene transcribed by the *veg* promoter of *B. subtilis* and translated from a synthetic *B. subtilis* ribosome binding site ($PvegbioA_{ec}$ cassette) in the presence of 6 g/l lysine, 3 g/l methionine (since the *E. coli* DAPA aminotransferase uses SAM and methionine is the precursor of SAM), and 1 g/l pimelic acid resulted in >90% conversion of KAPA to DTB and high levels of DTB production, 600-900 mg/l. Bioautography was used to confirm the absence of measurable amounts of KAPA. These data indicate that KAPA accumulation is at least partially caused by insufficient intracellular levels of the amino donor in fermentations with added pimelic acid. Increasing the concentration of lysine in the medium overcomes the KAPA to DAPA bottleneck and results in a significant improvement in DTB production.

Mutations can be introduced into BI282 that deregulate the lysine biosynthetic pathway (see Fig. 4). Fermentation experiments of two lysine analog (AEC) resistant mutants of biotin production strains showed improved DTB titers in the absence of added lysine. However, lysine is still limiting in these mutant strains. Additional mutations need to be added to further deregulate lysine biosynthesis if one wishes to eliminate the lysine feed. Such mutations include those that result in 1) deregulated expression of any or all of aspartokinases I, II, or III, 2) feedback resistant aspartokinases I, II, or III, 3) deregulated expression of diaminopimelate decarboxylase, 4) feedback resistant diaminopimelate

decarboxylase, or 5) any combination of the above (*Bacillus subtilis* and other Gram Positive Bacteria. (1993) A. Sowenstein J. Hoch, R. Losick (eds.) pp. 237-267. American Society for Microbiology. Washington, D.C.).

DAPA aminotransferase enzyme assay

The assay for DAPA aminotransferase is described by Eisenberg and Stoner in 1971 (*J. Bacteriol.* 108: 1135-1140). In this assay, the substrate KAPA is incubated with S-adenosylmethionine (SAM) in the presence of the cofactor pyridoxal 5'-phosphate and cell extract. We measured the amount of DAPA produced in a plate bioassay utilizing an *E. coli bio*A strain. Streptavidin (8 μg/ml) was added to the assay mix because extracts of many of the strains to be assayed contained significant amounts of biotin and dethiobiotin which fed the *E.coli* indicator strain used in the bioassay. Contamination by trace amounts of biotin and dethiobiotin was also removed from the KAPA preparation used as substrate by passing the material over an avidin-agarose column. The *E.coli bio*A109 strain (MEC1) was used to measure DAPA aminotransferase activity in the bioassay. This *E.coli bio*A strain, developed for the assay by Eisenberg, was reported to be many times more sensitive to DAPA than any other *bio*A mutant. Eisenberg's DAPA-sensitive strain was obtained from the *E.coli* Genetic Stock Center at Yale University.

B. subtilis DAPA aminotransferase does not utilize SAM as an amino donor.

A *B. subtilis* strain, BI282, engineered to overexpress *B. subtilis* BioA protein was assayed for DAPA aminotransferase activity: BI282 contains a $\mathbf{P}_{15}$ *bio* cassette amplified at the *bio* locus (described in Patent Application

0635572A2). A *B. subtilis* strain deleted for the *bio* operon, BI9 (Δbio::*neo*), was included as negative control. DAPA solutions of known concentration were spotted on the bioassay plates so that the amount of DAPA produced in each assay could be estimated. Measurable DAPA aminotransferase activity was seen in the BI282 extract, but not in the BI9 extract.

The enzyme reaction was approximately linear with time for at least 60 minutes. Using thin layer chromatography, the product of the reaction was shown to be DAPA. Enzyme activity was destroyed by boiling the extract, or freezing and thawing, although freezing the extract in the presence of 10% DMSO appeared to stabilize the enzyme. Activity was dependent on the presence of KAPA, but surprisingly, was not dependent on the presence of SAM. However, extracts assayed from *B. subtilis* strains lacking the native *bio*A gene but containing a *bio*A gene derived from either *E.coli* or S. *marcescens*, had DAPA aminotransferase activity dependent on the presence of exogenous SAM. We conclude that the *B. subtilis* DAPA aminotransferase utilizes a different amino donor than the *E.coli* or *S. marcescens* enzyme. Under the assay conditions used, the specific activity of the *B. subtilis* DAPA aminotransferase was found to be 100-fold lower than that of the *E.coli* or *S. marcescens* enzyme. This low specific activity could be due to limiting concentrations of the amino donor in the extract.

Identification of lysine as an amino donor for B. subtilis DAPA aminotransferase

To determine whether *B. subtilis* DAPA aminotransferase activity could be stimulated by addition of other amino donors to the reaction mix, various amino donors

were screened for their ability to stimulate enzyme activity *in vitro*. A cell free extract prepared from a *B. subtilis* strain deleted for the *bio* operon but containing multiple copies (4-6) of the *B. subtilis bioA* gene transcribed from a phage SP01-26 promoter with the cassette integrated at the *bpr* locus, BI611 ($\Delta bio::cat$, $bpr::[P_{26}bioA]_{4-6}$), was dialyzed to remove any endogenous levels of the amino donor and the extract was assayed in the presence of each of the standard amino acids and several other amine compounds. Of twenty-six compounds tested, only L-lysine hydrochloride (>98% pure) stimulated DAPA aminotransferase activity (***Table 1***). In a subsequent experiment, various lysine derivatives and analogs were tested for stimulation of activity (***Table 2***). The ability of a more purified preparation of L-lysine(>99% pure) to stimulate activity supported the conclusion that L-lysine is an authentic amino donor for the enzyme and argued against the possibility that the true amino donor was a contaminant in the lysine preparation. The ability of the L-lysine analog, (S)-2-aminoethyl-L-cysteine (AEC), to stimulate activity further supported the conclusion that L-lysine is the true amino donor for *B subtilis* DAPA aminotransferase. The structure of (S)-2-aminoethyl-L-cysteine is identical to L-lysine except that the γ carbon has been replaced by a sulfur atom.

The use of lysine as an amino donor by *B. subtilis* DAPA aminotransferase distinguishes the enzyme from other bacterial DAPA aminotransferases (from *E.coli*, *S. marcescens*, and *B. sphaericus*), which use SAM as an amino donor.

Kinetic studies of B. subtilis DAPA aminotransferase.

The kinetic properties of *B. subtilis* DAPA aminotransferase were investigated using a crude cell-free

extract prepared from BI611 (Δ*bio::cat*, *bpr::*[P_{26}*bioA*]$_{46}$). The production of DAPA from KAPA and lysine was shown to be approximately linear with time. The conversion of substrate to product was approximately 10 to 40% in 20 minutes. The total amount of DAPA produced during a standard 20 minute reaction was shown to be directly proportional to the amount of protein added to the reaction mix. The pH optimum for the transamination reaction was determined to be pH 8.6. A linear relationship was demonstrated between KAPA concentration (<20 μM) and specific activity, when the lysine concentration was kept constant at saturating levels (19mM) (***Figure 1***). Enzyme activity leveled off at KAPA concentrations between 20 μm and 80 μM, and inhibition of activity was observed at KAPA concentrations above 80 μM. Substrate inhibition by KAPA has also been demonstrated for *E. coli* DAPA aminotransferase by Eisenberg and Stoner (1971, *J. Bacteriol.* 108:1135-1140). The *E. coli* enzyme was subject to inhibition by KAPA at levels above 20 μM. An approximately linear relationship was demonstrated between *B. subtilis* bioA enzyme activity and lysine concentration (0-20 mM) when the KAPA concentration was held constant. The enzyme became saturated for lysine at concentrations between 20 and 40 mM.

The substrate inhibition of *B. subtilis* DAPA aminotransferase by KAPA provides evidence for a double displacement or ping-pong reaction mechanism, as has been shown for *E.coli* DAPA aminotransferase (Stoner et al., J. Biol. Chem. 250:4037-4043, 1975). Additional evidence supporting this conclusion is provided by the experiment presented in ***Figure 2***. KAPA concentration was varied at four different fixed lysine concentrations, and initial velocity data was collected and plotted in a double reciprocal form. The lines are approximately parallel in

the region of low KAPA concentration which is indicative of a ping-pong type reaction mechanism (Stoner et al., J. Biol. Chem. 250:4037-4043).

The apparent K_m values for lysine and KAPA for the *B. subtilis* DAPA aminotransferase reaction were determined to be in the range of 2-25 mM and 1-5 μM, respectively. The K_m of *E.coli* DAPA aminotransferase for KAPA was previously estimated to be 1.2 μM by Stoner and Eisenberg (1975, *J. Biol. Chem.* 250:4037-4043). The K_m for lysine was difficult to measure accurately because KAPA is a substrate inhibitor which presumably competes with lysine for binding to the active site at low lysine concentrations. Nevertheless, the apparent K_m of *B. subtilis* DAPA aminotransferase for lysine (2-25 mM) was significantly higher than the K_m of the purified *E. coli* enzyme for SAM (0.2 mM), as determined by Stoner and Eisenberg (1975, *J. Biol. Chem.* 250:4037-4043). While not wishing to be bound by a particular mechanism, it appears that the *B. subtilis* DAPA aminotransferase has a relatively high K_m for lysine, and that, in production strains which accumulate large amounts of KAPA, the *B. subtilis* DAPA aminotransferase is limited for lysine.

Fermentations of strains with enhanced* B. subtilis, E. coli, *or* S.marcescens *DAPA aminotransferase activity.

To test whether fermentations of the engineered biotin production strains were limited for the appropriate amino donors, a series of experiments were done in which lysine, methionine (the precursor to SAM), or lysine plus methionine were fed to fermentations of strains containing the appropriate *bioA* cassette and the level of KAPA-to-DAPA conversion was measured by vitamer bioassays and bioautography. These experiments were based in part on the hypothesis that the amino donor for the DAPA

aminotransferase became limiting during fermentation resulting in a build-up of KAPA.

All fermentations were carried out in computer controlled 14 liter Chemap fermentors utilizing a dissolved oxygen control, glucose-limited fed-batch fermentation strategy. The fermentations were performed using medium described in **Appendix 1**. Pimelic acid, lysine, and methionine were also batched and fed in the fermentations as indicated. The HABA-avidin displacement assay was used to determine the total amount of dethiobiotin and biotin in shake flask and fermentation samples. Coupling this chemical assay with bioassays (as described in EP 635572, and Tanaka et al., J. Micro. Methods 6:237-247, 1987) that determine biotin levels allows an additional determination of dethiobiotin production.

The HABA-avidin displacement assay is based on two facts: 1) HABA absorbs more strongly at 500 nm when bound to avidin than when free in solution and 2) DTB or biotin will quantitatively displace HABA from avidin. The description of this assay is presented as **Appendix 2**. The HABA assay is linear from 2 to 14 mg/l of dethiobiotin (DTB).

Total vitamers were measured as DTB equivalents in fermentation samples that had been acidified before autolaving to prevent KAPA breakdown. Total vitamers were determined as described in EP 0635572A2.

Lysine-fed fermentation of strains with enhanced *B. subtilis bioA* activity.

The effect of lysine feed on KAPA-to-DAPA production was studied by using strains, BI282 and BI603, that overexpress *B. subtilis* DAPA aminotransferase. BI282 overexpresses all biotin biosynthetic genes on a multicopy cassette ($\mathbf{P}_{15}$*bio*), integrated at the *bio* locus. BI603 is a

derivative of BI282 containing multiple copies of an additional *bioA* cassette (P_{26}*bioA*) integrated at the *bpr* locus which further increases the levels of DAPA aminotransferase. ***Table 3 (top)*** shows the optical densities, biotin and vitamer production of BI603 and BI282 grown with 1 g/l pimelic acid and 6 g/liter lysine in both the batch and feed. The total vitamers produced by BI603 and BI282 with lysine in the batch and feed were about 1300 mg/l and 1000 mg/l, respectively. The biotin production of the three fermentations were comparable (20-22 mg/l). The levels of HABA vitamers (biotin + DTB) in lysine-batched fermentations were sharply higher compared to previous fermentations without lysine. Typically BI282 and BI603 produced between 20-40 mg/liter HABA vitamers. Addition of lysine increased HABA vitamer production of BI603 to 570 mg/l and BI282 to 330 mg/l. Based on the biotin titers, most of the HABA vitamers produced from lysine feeding appeared to be in the form of dethiobiotin. Since biotin is formed from dethiobiotin, the HABA titer represents the total production level of dethiobiotin in the cells (for simplicity dethiobiotin production and HABA vitamer titers will henceforth be used interchangeably). Bioautographies of 30 hour fermentation samples of BI603 confirmed the accumulation of dethiobiotin and showed that DAPA was not accumulated in large quantities (approximately 10 mg/l).

***Methionine-fed fermentation of strains with enhanced* E. coli *or* S. marcescens BioA *activity*.**

The effect of methionine feed, the precursor to SAM, on the ~~conversation~~ of KAPA-to-DAPA was studied by fermentation of strains BI90 and BI96, expressing the *E. coli* or *S. marcescens* ATCC 31809 DAPA aminotransferase

enzymes, respectively. BI90 (*bio*::[P_{15}*bio*]$_{7-8}$ *sacB*::[P_{veg}*bioA*$_{ec}$]$_1$) and BI96 (*bio*::[P_{15}*bio*$_{7-8}$ *sacB*::[P_{veg}*bioA*$_{sm}$]$_1$) are derivatives of BI282 that contain a single-copy *E. coli* P_{veg}*bioA*$_{ec}$ or *S. marcescens* P_{veg}*bioA*$_{sm}$ cassette, respectively, integrated at the *sacB* locus. 1 g/l pimelic acid and 3 g/l methionine were added to both the batch and feed; exogenous lysine was not added to these fermentations in order observe the effect on KAPA-to-DAPA conversion by only the gram-negative DAPA aminotransferases. As a negative control, BI282, which does not contain an engineered gram-negative *bioA* gene, was also grown under identical conditions. As shown in ***Table 4***, total vitamer production of BI90, BI96, and BI282 was similar. Biotin production was slightly lower than usual (5-10 mg/l). The levels of HABA vitamers (biotin + DTB) in the methionine-fed fermentations of BI90 and BI96 were higher than the control BI282 fermentation. BI96 expressing the *S marcescens* ATCC 31809 P_{veg}*bioA*$_{sm}$ cassette produced 3-4-fold more HABA vitamers than BI282. BI90 expressing the *E coli* P_{veg}*bioA*$_{ec}$ cassette produced 5-6 fold higher levels of HABA vitamers. As with the previous lysine-fed fermentation of strains expressing the engineered *B. subtilis bioA* gene, most of the HABA vitamers were dethiobiotin. Addition of methionine to fermentation of strains with enhanced *E coli* or *S. marcescens* DAPA aminotransferase activity reduced the KAPA-to DAPA block presumably by increasing the level of SAM in the cell. Moreover, to the extent that the *B. subtilis* BioA enzyme synthesized from the engineered P_{15}*bio* operon in these strains is limited by insufficient lysine, conversion of KAPA-to-DAPA may increase when both lysine and methionine are fed to fermentations of BI90 or BI96.

Lysine and methionine-fed fermentations of strains with enhanced B. subtilis and E. coli BioA activities.

The effect of combining both lysine and methionine, the precursor for SAM, on the conversion of KAPA-to-DAPA in fermentations of a strain, BI90, expressing both *the E. coli* and *B. subtilis* DAPA aminotransferases, was studied by adding 1 g/l pimelic acid, 6 g/l lysine, and 3 g/l methionine to both the batch and feed. As control fermentations, BI603 was grown with or without 6 g/l lysine in the batch and feed. As shown in ***Table 5A***, BI603 without added lysine produce little HABA vitamers (30 mg/l) of which about 10 mg/l was dethiobiotin. However, with the addition of lysine, dethiobiotin production in BI603 increased more than 10-fold (510 mg/l). Moreover, fermentation of BI90 with both lysine and methionine resulted in almost two-fold more dethiobiotin (930 mg/l) than fermentation of BI603 with lysine alone. The range of dethiobiotin production in BI90 fermentations with lysine and methionine and 1 g/l pimelic acid was about 600-900 mg/l, but in all cases the majority of the KAPA was converted to DTB.

The level of KAPA remaining in these strains was confirmed by analyzing the 30 hour fermentation samples by bioautography using *E. coli ΔbioH* as the indicator (***Figure 3 and Table 5B***). In a separate bioautography using *E. coli* MEC1 indicator, DAPA was not detected in large quantities (15 mg/l for BI90 with lysine and methionine and 40 mg/l for BI603 with lysine; ***Table 6, bottom***), consistent with earlier lysine-fed fermentations of BI603 (***Table 3, bottom***).

Lysine-fed fermentations of strains with enhanced B. subtilis DAPA aminotransferase activity grown in Amberex based medium.

We examined the effect of different amounts of added lysine on biotin, DTB (HABA vitamers) and vitamer production in BI282 grown in fermentation medium with Amberex instead of VY (***Table 6***). Under these fermentation conditions, the addition of lysine at 7.5 g/l in batch and feed was sufficient to yield approximately 100% conversion of KAPA to DTB. Addition of higher levels of lysine in the feed (24.8 g/l) did not appear to be required. Fermentations with added lysine and pimelic acid produced about 10-fold more DTB (660-780 mg/l) than a fermentation of BI282 without lysine (60 mg/l). The fermentation without lysine produced 2-3 fold more biotin (12 mg/l) than fermentations with 10-fold higher levels of DTB (4-5 mg/l biotin).

Construction of BI282 derivatives that overproduce lysine.

We have also tried to increase the cell's lysine pool by an alterative method, namely boosting the internal lysine biosynthesis capacity. Strains of *Brevibacter and Corynebacter* have been developed to product about 80 g/l lysine, so it should be possible to engineer *B. subtilis* to overproduce lysine to the extent necessary to stimulate DTB synthesis. There are two basic approaches to take, 1) collect known mutants that are deregulated for lysine biosynthesis and move the relevant mutations into a biotin producing strain, and 2) isolate mutants deregulated for lysine production by selecting for lysine analog resistance directly in a biotin producing strain background.

Known lysine deregulated mutants of B. subtilis.

The biosynthetic pathway from aspartate to lysine for *B. subtilis* is outlined in ***Figure 4***. The two regulated steps are the first step, catalyzed by aspartokinase, and the last step, which incidentally is the first step

committed solely to lysine, catalyzed by diaminopimelate (DAP) decarboxylase. Both steps are regulated by feedback inhibition and at the level of gene expression. A summary of the regulated enzymes is given in ***Table 7***.

Four types of mutations leading to deregulated lysine synthesis are known, 1) a DAP resistant aspartokinase I, 2) a constitutive aspartokinase II, 3) a lysine resistant DAP decarboxylase, and 4) an undefined S-2-aminoethyl-L-cysteine (AEC) resistant mutation that is unlinked to any of the known lysine genes. These known mutations are summarized in ***Table 7***. The last three all have an AEC resistant phenotype, and so each could be moved into a biotin production strain by transduction, transformation, or congression.

Isolation of lysine overproducers directly in biotin producing strain background.

Three out of the four classical cases of lysine deregulated mutants were isolated by selecting for lysine analog resistance. *B. subtilis* strains, PY79 (Youngman et al., Plasmid 12:1-9, 1984), BI282, and BI603 were tested for sensitivity to four lysine analogs, on minimal medium with no additive, with threonine, or with DAP plus threonine. The purpose of the additives was to focus the selection on the *lysC* gene, that encodes the lysine sensitive aspartokinase II. The only analog that inhibited growth under any conditions was AEC. All three strains behaved similarly; all were sensitive to AEC in all three media.

Spontaneous AEC resistant mutants were isolated from PY79, BI282, and BI603. The mutations in these strains are most likely to be *lysC* constitutive mutants, because according to the literature, most AEC resistance mutants are of that type. Eleven mutants from each parent were tested

for lysine secretion in a minimal medium. The assay used was a biological assay using *B. subtilis* 1A615 (*trpC2 lys*::Tn917) as an indicator strain. None of the parent strains secreted lysine detectable by the assay (<2 mg/l). However, 10 out of 11 PY79 mutants secreted lysine in the range of 20 to 70 mg/l, one BI282 mutant secreted 30 mg/l and one BI603 mutant secreted 26 mg/l lysine. The BI282 and BI603 mutants, called BI641 and BI642, were then tested for biotin production in the fermentor without a lysine feed and compared to BI282 with a lysine feed. As shown in ***Tables 5, 6, and 8***, BI641 and BI642 produced a higher level of DTB than the respective parent strains in the absence of lysine, but not as much as when 6 g/l lysine was fed. Lysine biosynthesis can be further deregulated by introducing a second lysine deregulating mutation as described above.

Deposit statement

The subject cultures listed below are deposited under conditions that assure that access to the cultures will be available during the pendency of the patent application disclosing them to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. 1.14 and 35 U.S.C. 122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., they will be stored with all the care

necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposits, and in any case, for a period of at least 30 (thirty) years after the date of deposit or or the enforceable life of any patent which may issue disclosing the cultures plus five years after the last request for a sample from the deposit. The depositor acknowledges the duty to replace the deposits should the depository be unable to furnish a sample when requested, due to the condition of the deposits. All restrictions on the availability to the public of the subject culture deposits will be irrecovably removed upon the granting of a patent disclosing them.

Strain	ATCC No.
BI 90	[illegible]
BI 96	[illegible]
BI 603	[illegible]
BI 641	[illegible]
BI 642	[illegible]
BI 482	55574

Other embodiments are within the following claims.

TABLES

FOR

UNITED STATES LETTERS PATENT

TITLE: **OVERCOMING DAPA AMINOTRANSFERASE BOTTLENECKS IN BIOTIN VITAMERS BIOSYNTHESIS**

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9 PAGES OF TABLES

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Lisa G Gray

Lisa G Gray

TABLE 1

Amino donor tested	Stimulation of activity	Amino donor tested	Stimulation of activity
none	–	L-glutamic acid	–
L-methionine	–	L-lysine	+
L-aspartic acid	–	L-tryptophan	–
L-asparagine	–	L-valine	–
L-tyrosine	–	L-leucine	–
L-cysteine	–	L-alanine	–
L-proline	–	L-isoleucine	–
L-serine	–	L-ornithine	–
L-glycine	–	L-homoserine	–
L-glutamine	–	DL-homocysteine	–
L-threonine	–	spermine	–
L-histidine	–	S-adenosyl-L-methionine	–
L-phenylalanine	–	S-adenosyl-L-homocysteine	–
L-arginine	–		

TABLE 2

Compound added to extract	DAPA aminotransferase specific activity (nmoles/min/mg)
none	0
L-lysine (>98%)	0.76
L-lysine (>99%)	0.56
D-lysine (>98%)	0.19
DL-lysine (>98%)	0.35
Nα-acetyl-L-lysine	0
Nε-acetyl-L-lysine	0
Nε-methyl-L-lysine	0
gly-lys	0
lys-gly	0
(S)-2-aminoethyl-L-cysteine	0.48
diaminopimelic acid	0

TABLE 3

Fermentation #/ Strain	Lysine (6 g/liter) Batch	Feed	Time (hr)	OD_{600}	Total Vitamers (mg/liter)	Biotin (mg/liter)	HABA Vitamers (mg/liter)	Calculated DTB (mg/liter)
B160/BI603	+	–	24	150	740	16	330	314
B160/BI603	+	–	30	160	950	22	400	378
B161/BI603	+	+	24	140	1100	14	420	406
B161/BI603	+	+	30	160	1290	20	570	550
B162/BI282	+	+	24	132	1100	10	220	210
B162/BI282	+	+	30	140	1000	22	330	308

Vitamer Breakdown

Fermentation #/ Strain	Lysine (6 g/liter) Batch	Feed	Time (hr)	KAPA (mg/liter)	DAPA[a] (mg/liter)	DTB (mg/liter)	Biotin (mg/liter)	Total (mg/liter)
B161/BI603	+	+	30	710	10	550	20	1290

[a] Estimated from bioautography of a an acid autoclaved sample using *E. coli* MEC1 indicator.

TABLE 4

Fermentation #/ Strain	Time (hr.)	OD_{600}	Total Vitamers (mg/liter)	Biotin (mg/liter)	HABA Vitamers (mg/liter)	Calculated DTB (mg/liter)
BI63/BI90	24	150	760	8	126	118
BI63/BI90	30	160	720	9	145	136
BI64/BI96	24	170	830	9	84	75
BI64/BI96	30	160	850	10	88	78
BI65/BI282	24	140	610	5	17	12
BI65/BI282	30	150	590	6	25	19

TABLE 5A

Fermentation #/ Strain	Batch and Feed Lys (6 g/liter)	Batch and Feed Met (3 g/liter)	Time (hr)	OD_{600}	Total Vitamers (mg/liter)	Biotin (mg/liter)	HABA Vitamers (mg/liter)	Calculated DTB (mg/liter)
B166/BI603	–	–	24	150	800	20	30	10
B166/BI603	–	–	30	155	600	21	30	9
B167/BI603	+	–	24	143	800	6	460	454
B167/BI603	+	–	30	166	870	5	510	506
B168/BI90	+	+	24	128	800	5	890	885
B168/BI90	+	+	30	165	1000	5	930	925

TABLE 5B

Vitamer Breakdown

Fermentation #/ Strain	Batch and Feed: Lys (6 g/liter)	Batch and Feed: Met (3 g/liter)	Time (hr)	KAPA (mg/liter): [a]	KAPA (mg/liter): [b]	DAPA[c] (mg/liter)	DTB (mg/liter)	Biotin (mg/liter)	Total (mg/liter)
B166/BI603	–	–	30	570	470	0	9	21	600
B167/BI603	+	–	30	320	250	40	505	5	870
B168/BI90	+	+	30	55	60	15	925	5	1000

[a] Calculated by subtracting DAPA, DTB, and biotin titers from total vitamers.

[b] Estimated from bioautography of acid autoclaved samples using *E. coli ΔbioH* indicator.

[c] Estimated from bioautography of acid autoclaved samples using *E. coli* MEC1 indicator.

TABLE 6

Run/Strain (Drug)	Lysine (g/liter) Batch	Lysine (g/liter) Feed	Time (hr.)	OD_{600}	Total Vitamers (mg/liter)	HABA Vitamers (mg/liter)	Biotin (mg/liter)	%KAPA to DTB conversion (mg/liter)
B235/B1282 (CAM60)	7.5	24.8	24	107	590	600	4	100
			30	122	830	660	4	89
B236/B1282 (CAM60)	---	---	24	123	410	40	11	10
			30	130	450	60	12	13
B237/B1282 (CAM60)	7.5	7.5	24	115	630	780	4	100
			30	124	670	750	5	100

*Batch medium (Amberex) contained 1 g/l pimelic acid and the indicated lysine amount; Feed medium contained 1 g/l pimelic acid and the indicated lysine amount.

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Table 7

Enzyme	Type of Mutation	Gene	Map Location	Inhibitor	Corepressor	Decrease in stationary
Aspartokinase I	DAP^r	*dapG*	149	DAP	none known	no
Aspartokinase II	constitutive	*lysC*	252	lysine	lysine	yes
Aspartokinse III	---	---	---	lysine & threonine	threonine	yes
DAP decarboxylase	lys^r	*lysA*	210	lysine	lysine & ?	yes
---	---	*aecB*	282	---	---	---

TABLE 8

Fermentation #/ Strain	Lysine (6 g/liter) Batch	Lysine (6 g/liter) Feed	Time (hr)	OD600	Total Vitamers (mg/liter)	Biotin (mg/liter)	HABA Vitamers (mg/liter)	Calculated DTB (mg/liter)
B190/BI282	+	+	24	84	240	6	270	264
B190/BI282	+	+	30	125	390	7	360	353
B191/BI641 (BI282*aec7*)	–	–	24	74	470	5	130	125
B191/BI641 (BI282*aec7*)	–	–	30	129	500	6	144	138
B192/BI642 (BI603*aec11*)	–	–	24	86	540	4	160	156
B192/BI642 (BI603*aec11*)	–	–	30	120	560	5	110	105